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Patterns of IgE sensitization in house dust mite-allergic patients: implications for allergen immunotherapy

T. Batard¹, V. Baron-Bodo¹, A. Martelet¹, M. Le Mignon¹, P. Lemoine¹, K. Jain¹, S. Mariano¹, S. Horiot¹, H. Chabre¹, C. Harwanegg², C.A. Marquette³, B.P. Corgier³, W. T. Soh⁴, P. Satitsuksanoa⁴, A. Jacquet⁴, F.T. Chew⁵, E. Nony¹, P. Moingeon¹

¹ Research and Development, Stallergenes, Antony, France

² Thermo Fisher Scientific ImmunoDiagnostics / Phadia Austria GmbH, Vienna, Austria

³ AXO Science, Villeurbanne, France

⁴ Chulalongkorn University, Bangkok, Thailand

⁵ National University of Singapore, Singapore

Correspondence

Philippe Moingeon, Research and Development, Stallergenes, 6 rue Alexis de Tocqueville, 92183 Antony Cedex, France. Tel.: 33.(0)1.55.59.49.57 - Fax: 33.(0)1.55.59.21.02

Email: pmoingeon@stallergenes.com

Abstract

Background

Understanding patterns of IgE sensitization in *Dermatophagoides*-allergic patients living in various geographical areas is necessary to design a product suitable for worldwide allergen immunotherapy (AIT).

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Methods

Using a *HIFI Allergy* customized microarray assay, IgEs specific for 12 purified allergens from *Dermatophagoides pteronyssinus* or *D. farinae* were assessed in sera from 1302 house dust mite (HDM)-allergic patients living in various areas. Comprehensive mass spectrometric (MS) analyses were conducted to characterize HDM extracts, as well as purified bodies and feces.

Results

Patterns of IgE reactivity to HDM allergens are comparable in all cohorts of patients analysed, encompassing adults and 5-17 years old children, as well as American, Canadian, European and Japanese patients. Overall, > 70% and > 80% of HDM-allergic patients are sensitized to group 1 and group 2 allergens, respectively, from *D. pteronyssinus* and/or *D. farinae* species. Furthermore, 20 to 47% of patients also have IgEs to allergens from groups 4, 5, 7, 13, 15, 21 and 23. All patients have IgEs to allergens present in mite bodies and feces. MS-based analyses confirmed the presence of mite allergens recorded by IUIS in *D. pteronyssinus* and *D. farinae* extracts, with groups 2, 8, 10, 11, 14 and 20 prominent in bodies and groups 1, 6, 18 and 23 well represented in feces.

Conclusions

Mite-specific AIT should rely upon a mixture of *D. pteronyssinus* and *D. farinae* extracts, manufactured from both feces and bodies. Such a combination is appropriate to treat children and adult *Dermatophagoides*-allergic patients from Asia, Europe and North America.

Short title

House dust mite seroepidemiology: implications for AIT

Key words

Allergen immunotherapy, *Dermatophagoides*, house dust mite, IgE sensitization

Abbreviations

AIT: Allergen immunotherapy

CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

ESI-Q-ToF: Electrospray ionization quadrupole time-of-flight

HDM: House dust mite

IAU: Intensity arbitrary unit

ISAC: Immunosolid-phase allergen chip

ISU: ISAC standardized unit

LC: Liquid chromatography

MS: Mass spectrometry

MS/MS: Tandem MS

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2D-DiGE: Two-dimensional differential gel electrophoresis

Introduction

House dust mites (HDM) represent a major source of allergens affecting more than 500 million people throughout the world (1-3). Although mites encompass more than 40,000 distinct species, the two common Pyroglyphidae *Dermatophagoides pteronyssinus* and *D. farinae* species are responsible for more than 90% of HDM allergies worldwide (2, 3). In sensitized patients, such HDM can induce allergic rhinitis, asthma and/or atopic dermatitis. Whereas allergy symptoms associated with mite exposure can be alleviated by pharmacological drugs, allergen immunotherapy (AIT) is the only etiologic treatment providing sustained efficacy for moderate to severe allergic rhinitis to HDM (1, 3, 4). Some approaches based on recombinant allergens or peptides are under development (5-8), but AIT is currently based on the repeated administration of aqueous mite allergen extracts prepared from either bodies or whole mite cultures, the latter comprising as well fecal particles (9-15). Historically, mites have been grown using various culture media containing either human scales, dried daphnia, cereals, mold cultures, as well as fish or dog food (16). However, in order to manufacture pharmaceutical-grade extracts for the treatment of allergic patients, mites cannot be cultured using a medium containing any protein of human or animal origin. In this context, we have developed a well-defined culture medium made of synthetic amino acids, wheat germ and yeast, which allows to produce HDM with similar

characteristics to the ones grown on human scales (17).

Since AIT aims to reeducate the immune system in a specific manner by eliciting tolerance to the sensitizing allergens (18, 19), the design of AIT products must take into account the patterns of IgE sensitization of allergic patients, with as a final goal to recapitulate at a molecular level (i.e. in terms of allergen composition) natural exposure conditions. In this context, we conducted seroepidemiological studies in *Dermatophagoides*-allergic pediatric and/or adult patient populations from Canada, Europe, Japan and the United States. In light of the obtained data, together with an extensive molecular characterization of HDM extracts, we conclude that immunotherapy should rely upon extracts made from both *D. pteronyssinus* and *D. farinae*, providing allergens expressed in bodies and fecal particles from the two species.

Materials and methods

Analysis of IgE reactivity in sera from house dust mite allergic patients

Blood samples were obtained after informed consent from 503 European adults living in 7 distinct countries (i.e. Czech Republic, France, Germany, The Netherlands, Poland, Slovakia and Spain) (20), 423 pediatric (5 to 17 years old) patients originating from 9 distinct countries (i.e. Denmark, France, Germany, Hungary, Ireland, Romania, Slovakia, Spain and Ukraine), as well as 350 HDM -allergic Canadian adult patients residing in Ontario with an history of HDM-related allergic rhinoconjunctivitis for at least 1 year, a positive skin prick test to *D. pteronyssinus* and/or *D. farinae* with a wheal diameter > 3 mm and IgE titers to *D. pteronyssinus* and/or *D. farinae* > 0.7 kU/L, as determined using the Immulite 2000 Immunoassay system (Siemens, Munich, Germany) or ImmunoCAP assay (Thermo Scientific, Uppsala, Sweden). Titers of serum IgE to Der p 1, Der p 2, Der f 1 and Der f 2, as well as Der p 5, Der p 7 and/or Der p 10, were assessed in parallel using ImmunoSolid-Phase Allergen Chip (ISAC) assays with natural or recombinant purified allergens (Thermo Scientific), as described elsewhere (21). Results were expressed in ISAC Standardized Units (ISU), and considered positive when anti-allergen IgE levels were > 0.3 ISU. Whereas our focus was on allergic rhinoconjunctivitis, a small percentage (i.e. 7.9%) of the patient population had concomitant atopic dermatitis or eczema.

In addition, sera from 28 HDM-allergic Japanese patients were purchased from Trina Bioreactives (Nänikon, Switzerland) without any specific requirement regarding the living place

of the donors. These sera were selected on the basis of a clinical history suggestive of HDM allergy and a positive IgE reactivity to a *D. pteronyssinus* extract with specific IgE levels > 3.5 kU/L, measured by ImmunoCAP. Sera from 35 HDM-sensitized US individuals were also purchased from PlasmaLab (Everett, WA, USA) without any specific requirement regarding the living place of the donors. Such sera were selected on the basis of a positive IgE reactivity to *D. pteronyssinus* and/or *D. farinae* extracts, with specific IgE levels > 1 kU/L, as measured by ImmunoCAP. No information was available regarding potential mite-related symptoms in this group. Serum samples from 4 individuals unsensitized to HDM allergens were included as negative controls in the *HIFI Allergy* microarray experiments.

Multiplexed IgE quantification assays were carried out using the high-throughput *HIFI* Assay platform (AXO Science, Villeurbanne, France) with an EVO100 equipment (Tecan, Männedorf, Switzerland) and a 96-head automated washer as described elsewhere (22) and summarized in Supplementary Methods. Highly significant correlations ($p < 0.0001$) were found between IgE levels measured by the *HIFI Allergy* assay and ImmunoCAP or ISAC methods, for all allergens tested (Table S1). A direct comparison of results obtained with the three assays confirmed that the cut-off value for a positive signal with the *HIFI Allergy* array is around 0.35 kU/L or 0.3 ISU (data not shown).

Mite extracts, bodies and feces, natural and recombinant purified mite allergens

Mites of the *D. pteronyssinus* and *D. farinae* species were grown for 75 and 85 days, respectively and harvested as described elsewhere [17]. *D. pteronyssinus* or *D. farinae* source materials were incubated for 24 h +/- 2 h at +5°C +/- 3°C in 4 g/L ammonium bicarbonate. Extracts were obtained after centrifugation, clarifying filtration of the supernatant on 0.22-µm membrane filters, concentration and diafiltration on a 5-kDa cut-off membrane, addition of 2% (mass/mass) mannitol, freeze-drying and sieving. Mite bodies were purified by using a flotation method whereas feces were purified by sequential sieving as described elsewhere (23) and summarized in Supplementary Methods. 2D-DiGE and mass spectrometry (MS) analyses of proteomes and allergomes are described in Supplementary Methods. Group 1 allergens were quantified using an ELISA assay (Indoor Biotechnologies, Cardiff, UK) and group 2 allergens were measured by MS as described in Supplementary Methods.

The WHO/IUIS allergen nomenclature (available on www.allergen.org) was used in the present study. Production and purification of natural (n) Der p 1, Der f 1, Der p 2, Der f 2, Der p 4 molecules and recombinant (r) Der p 5, Der p 7, Der p 10, Der p 13, Der p 15, Der p 21, Der p 23 allergens are described in Supplementary Methods. The quality of these purified allergens was confirmed as described in Supplementary Methods and Fig. S1.

Statistical analyses

Analyses were performed with the Microsoft Excel software. Correlation analyses were performed by using the non-parametric Spearman test with the GraphPrism 5 software (GraphPad Software, Inc, La Jolla, USA); p-values < 0.05 were considered significant.

Results

Patterns of IgE sensitization to major and minor allergens from *D. pteronyssinus* and *D. farinae* in HDM-allergic patients

In a first set of experiments, we compared the IgE reactivity in sera from HDM-allergic children (5-17 year old, n = 416) and adults (n = 503) using group 1 and group 2 allergens purified from both *D. pteronyssinus* and *D. farinae* species. Results obtained using the ISAC assay demonstrated a comparable pattern of IgE reactivity with mite major allergens in the two cohorts (**Fig. 1**). Specifically, both group 1 and group 2 allergens from *D. pteronyssinus* and *D. farinae* are relevant in the two populations, since 79% of children and 66% of adults have IgEs recognizing group 1 allergens (i.e. Der p 1 and/or Der f 1), whereas 92% of children and 81% of adults have IgEs directed to group 2 allergens (i.e. Der p 2 and/or Der f 2) (**Fig. 1A**). Overall, 53-70% of patients exhibit serum IgE reactivity to all of these four major allergens (not shown). Noteworthy, 6-7% of patients have IgEs recognizing group 1 but not group 2 allergens, whereas 19-22% of them have IgEs reacting with group 2 but not group 1 allergens (**Fig. 1B**). We estimate that $\geq 5\%$ of children and adults have IgEs reactive with major allergens from only one of the two mite species.

We subsequently analysed IgE sensitization profiles in a total of 1,302 sera from *Dermatophagoides*-allergic patients from Canada, Japan and the USA, including as well the ones from Europe (adults and children) tested above. Using a customized multiplexed (*HIFI Allergy*) microarray assay described in Supplementary Methods, we assessed not only the IgE response to group 1 and 2 major allergens, but also to minor or mid-potency allergens belonging to groups 4, 5, 7, 10, 13, 15, 21 and 23 obtained as purified natural or recombinant molecules. Analyses conducted with the *HIFI Allergy* assay established that patterns of IgE sensitization assessed both in terms of prevalence and relative contribution to total IgE reactivity, are highly comparable in the different cohorts of patients analysed, irrespective of their geographical origin (**Fig. 2A, B**). Altogether, those analyses confirmed that allergens from groups 1 and 2 from *D. pteronyssinus* and *D. farinae* represent major allergens, with more than 65% of tested patients having IgEs to each of them (**Fig. 2A**). Furthermore, group 4, 5, 7, 13, 15, 21 and 23 allergens might be important for selected patients, as they are recognized by IgEs from $\geq 20\%$ of patients in the various populations (**Fig. 2A**). In contrast, irrespective of the population considered, $\leq 11\%$ of patients displayed serum IgEs to Der p 10 (**Fig. 2A**), establishing the latter as a very minor allergen. In light of these results, we subsequently assessed the pooled data obtained from the 1302 patients regarding the overall prevalence of IgE reactivity with allergens found predominantly in either mite bodies or feces, respectively (see below). As shown in **Fig. 2A**, a majority of patients are sensitized to allergens well represented in mite bodies (e.g. Der p 2 and Der f 2), but also prominent in fecal particles (such as Der p 1, Der f 1 and Der p 23).

Characterization of major and minor allergens in *D. pteronyssinus* and *D. farinae* whole extracts, bodies and feces

Having documented a standard pattern of IgE sensitization for HDM-allergic patients of various origins, we investigated the allergen composition of *D. pteronyssinus* and *D. farinae* extracts intended for AIT. We first compared extracts from the two species using a 2D-differential gel electrophoresis (2D-DiGE) analysis of mixed proteins from *D. pteronyssinus* highlighted in green and those from *D. farinae* in red (**Fig. 3A-C**). Allergens from the two species were localized by spot picking and MS/MS sequencing (**Fig. 3C**, group 1: white arrows, group 2: blue arrows). This comparative analysis confirmed that proteomes and allergomes from *D. pteronyssinus* and *D. farinae* species are different, with only very limited overlay (yellow spots) in merged pictures

(Fig. 3C). A comprehensive MS analysis of those mite extracts subsequently demonstrated the presence of group 1-11, 14, 15, 18, 20, 21 and 23 allergens in *D. pteronyssinus* extracts (Table 1) and of group 1-3, 6-8, 10, 11, 13-16, 18, 20-22, 25-33 allergens in *D. farinae* extracts (Table 2). These results obtained by LC-MS/MS were further corroborated by immunoblot, using available allergen-specific antisera confirming the presence of mite allergens (including group 1 to 10, as well as group 13) in *D. pteronyssinus* [17] and *D. farinae* extracts (Fig. S2).

In order to determine the origin of these allergens, a characterization of the allergomes was further performed in bodies and feces purified by flotation and sedimentation, respectively, from cultured *D. pteronyssinus* or *D. farinae* mites. Using 2D-DiGE, we first confirmed that proteomes from bodies (protein spots highlighted in green) and feces (spots highlighted in red) are significantly different for both *D. farinae* (Fig. 3D-F) and *D. pteronyssinus* (Fig. 3G-I), with only partial overlay (yellow spots) when the figures were merged. MS-based analyses confirmed that mite feces from *D. pteronyssinus* and/or *D. farinae* are rich in groups 1, 6, 18 and specifically for *D. pteronyssinus* in group 23 (Table 1, Table 2). In contrast, bodies contain more group 2, 8, 10, 11, 14 and 20 allergens in both *D. pteronyssinus* and/or *D. farinae* species. Der f 18 previously identified in the gut using immunostaining (24) was also detected in feces by MS.

Characterization of culture medium-derived proteins in *D. pteronyssinus* and *D. farinae* extracts

Lastly, we investigated the potential presence of residual components from the mite culture medium used to grow *D. pteronyssinus* and *D. farinae* in allergen extracts. To this end, enzymatic digests of *D. pteronyssinus* and *D. farinae* extracts were analyzed versus an in-house data base encompassing mite allergens as well as *Saccharomyces cerevisiae* and *Triticum aestivum* proteins. Whereas numerous peptides originating from mite proteins (2,200 and 1,454 peptides for *D. pteronyssinus* and *D. farinae* extracts, respectively) were identified in those analyses, only 13 and 9 peptides found in *D. pteronyssinus* and *D. farinae* extracts, respectively, were derived from yeast or wheat germ, with none of those corresponding to known wheat or yeast allergens. No gluten was detected in whole mite extracts.

Discussion

Mite allergic patients in temperate countries are classically co-exposed and co-sensitized to allergens from both *D. pteronyssinus* and *D. farinae* species [2, 3]. Thus, in order to design allergen products suitable to treat HDM-allergic patients in various parts of the world, it is critical to understand the patterns of IgE sensitization to allergens from such common *Dermatophagoides* species. To this aim, we performed a seroepidemiological study enrolling 1,302 *Dermatophagoides*-allergic patients from Canada, Europe, Japan and US.

This study established that (i) patterns observed in 5-17 year old children and adults are similar, (ii) all patients exhibit comparable patterns of IgE sensitization, irrespective of their geographical location. Among mite allergens, group 1 and 2 allergens are clearly the most important ones, both in terms of prevalence and relative contribution to total IgE reactivity against HDM allergens. Our results are broadly in agreement with the literature (3, 25-29) with evidence that many additional minor allergens from *Dermatophagoides* are recognized by IgEs from HDM-allergic patients, including group 4, 5, 7, 13, 15, 21 and 23 allergens (**Fig. 2**). In our study, 46.5% of patients have serum IgEs specific to Der p 23, but the latter only contributes to 6% of total mite-specific IgE reactivity, with variability among patients suggesting that it may be clinically relevant for selected patients. The low prevalence of IgE reactivity to Der p 10 observed herein confirms previous reports concluding that it represents a minor allergen (25, 30, 31). Some differences are observed between US patients and other cohorts in terms of IgE reactivity with group 4 and 23 allergens. Those differences could be explained by both a few outliers, the small size of the US cohort as well as differences in the patients' selection.

Interestingly, while most patients have IgEs recognizing group 1 and/or group 2 allergens from both *D. pteronyssinus* and *D. farinae*, we estimate that $\geq 5\%$ of them have IgEs directed to group 1 and/or group 2 allergens from only one of those mite species (**Fig. 1**). This confirms that despite a well known amino acid sequence homology, each of Der p 1, Der f 1, Der p 2 and Der f 2 bears species-specific IgE epitopes. Although not shown, we also observed similar differences in terms of IgE reactivity between allergens from the two mite species, for each of the group 3 to 9 and 21 minor allergens (data not shown). Interspecies differences in the

immunogenicity of mite allergens have been reported by others, both in terms of T and B cell epitopes (32-34). Altogether, these results strongly suggest that when patients have IgEs to both *D. pteronyssinus* and *D. farinae* allergens, this reflects not only IgE cross-reactivity, but also a *bona fide* co-exposure and sensitization (**Fig. 1B**). From those observations, we infer that a proper product for AIT should contain both *D. pteronyssinus* and *D. farinae* extracts, in agreement with our demonstration in a murine experimental model that such a combination is indeed more efficacious than each of *D. pteronyssinus* or *D. farinae* single extracts for alleviating asthma symptoms in animals exposed to the two mite species (35).

Using MS to characterize aqueous extracts from *D. pteronyssinus* and *D. farinae*, we confirm the presence of all allergens currently recognized by IUIS (with the exception of Der f 17 and Der f 24) (**Table 1, Table 2**). This result is consistent with our previous analysis by immunodot blotting establishing that *D. pteronyssinus* extracts contain Der p 3 to Der p 10, Der p 13 and Der p 14 (17). Using the same method, we confirm herein that *D. farinae* extracts contain Der f 3 to Der f 10 as well as Der f 13 (Fig. S2). We document as well the presence of the newly characterized group 25-33 allergens (36) by MS in *D. farinae* extracts (**Table 1**). In the absence of validated assays to measure these allergens, quantitative data regarding such minor allergens are not yet available. We established that some allergens are predominantly associated with mite feces, such as allergens from group 1, 6, 18 and 23 allergens, while others are more abundant in mite bodies, including group 2, 8, 10, 11, 14 and 20 allergens, in agreement with other studies (23, 37, 38). Interestingly, we detected group 2 and group 18 allergens by MS, not only in bodies as previously described, but also in feces, possibly because MS provides a more comprehensive allergen detection encompassing all isoallergens, whether present in a native or denatured form, when compared with immunological methods. Given that most patients have IgEs to allergens expressed in bodies and feces, we conclude that in order to provide a broad spectrum of mite allergens and derived isoforms, immunotherapy should be performed using extracts made from both bodies and feces of the two *D. pteronyssinus* and *D. farinae* species.

The potential presence in mite extracts of culture medium-derived components is a theoretical concern, most particularly if it were to include potential allergens. By using western blotting and HPLC, we previously demonstrated that *D. pteronyssinus* and *D. farinae* extracts obtained using our culture conditions do not contain potential marker proteins nor allergens from wheat germ and yeast included in the culture medium (17). By using sensitive nanoLC-MS/MS, we corroborate those findings, with only few peptides corresponding to non-allergenic proteins from yeast or wheat germ detected in *D. pteronyssinus* and *D. farinae* extracts. The latter is also in line with our previous observation that no IgE sensitization to culture components was observed over a 2-year follow-up period when HDM-allergic patients were treated with sublingual tablets containing extracts from the two mite species (39).

Altogether, seroepidemiological results combined with comprehensive characterization data of HDM allergen extracts lead to the conclusions that (i) the same product can be used for AIT of *Dermatophagoides*-allergic patients, irrespective of their age and geographical location, (ii) this product should rely upon a mixture of *D. pteronyssinus* and *D. farinae* extracts, produced with corresponding source materials encompassing both bodies and feces.

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Conflicts of interest

This work was funded by Stallergenes. TB, VB-B, AM, MLM, PL, KJ, SM, SH, HC, EN and PM are employed by Stallergenes. The other authors declare no competing financial interest.

Authors' contributions

TB wrote the manuscript, supervised part of the people involved in the study, and interacted with others. VBB supervised the seroepidemiological study. AM and MLM performed LC-MS analyses. PL purified mite bodies and feces and characterized allergens. KJ performed the 2D-

DiGE experiments and characterized allergens. SH contributed to seroepidemiological studies. SM, HC, WTS, PS and AJ produced and purified allergens. CH supervised the ISAC seroepidemiological studies. CAM and BPC performed analyses conducted using *HIFI Allergy*. FTC supervised the dot blot analysis. EN and PM supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Material and methods.

Figure S1. SDS PAGE analysis of purified allergens used in the *HIFI Allergy* customized multiplexed mite microarray.

Figure S2. Immunodot blot analysis of representative *D. farinae* extracts.

Table S1. Correlations between allergen-specific IgE levels measured by using either ImmunoCAP, ISAC assay, or *HIFI Allergy* customized multiplexed microarray.

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FIGURE LEGENDS

FIGURE 1: Frequencies of IgE responses to group 1 and group 2 allergens from *D. pteronyssinus* and *D. farinae* in pediatric and adult patient populations

IgE responses were measured by using the ISAC assay in sera from 503 European adult and 416 pediatric patients allergic to HDM. Results are presented either globally (A) or in combination (B). For instance, “Der p 1+ / Der f 1-” indicates patients who have IgEs to Der p 1 but not to Der f 1, and “group 1+ / group 2-” indicates patients who have IgEs to Der p 1 and/or Der f 1 but not to either Der p 2 or Der f 2.

FIGURE 2: Frequencies and relative contribution of IgE responses to HDM allergens

IgE responses were measured in 466 European adults, 423 European children (aged 5-17 years), 350 Canadian adults, 35 American adults and 28 Japanese adults allergic and/or sensitized to *Dermatophagoides* species by using a customized multiplexed mite microarray (*HIFI Allergy*). (A) Patterns of IgE sensitization and (B) contribution to IgE binding of each purified allergen from *D. pteronyssinus* relative to total IgE reactivity against all *D. pteronyssinus* allergens measured with the *HIFI Allergy* microarray. Data are shown as means + SEMs.

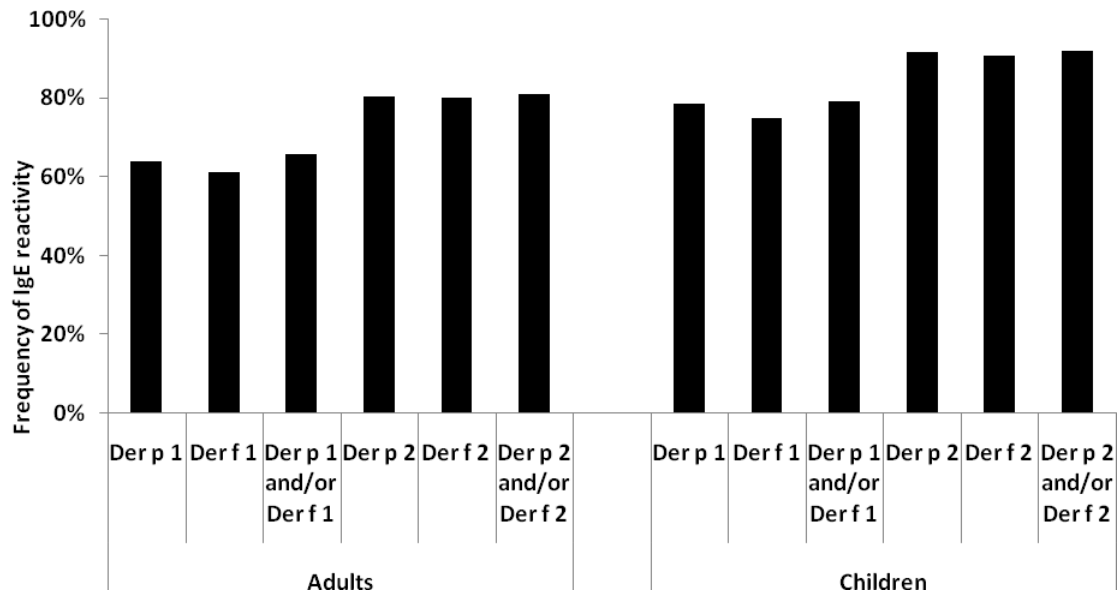
FIGURE 3: Comparative analyses of mite proteomes by 2D-DiGE

Proteins from *D. pteronyssinus* and *D. farinae* were labeled using distinct fluorescent dyes, mixed and analysed by 2D-gel electrophoresis. 2D gels from (A) whole *D. farinae* extract (in red), (B) whole *D. pteronyssinus* extract (in green) and (C) the overlay of both. 2D gels from *D. farinae* (D) purified feces (red), (E) purified bodies (green) and (F) the overlay of both. 2D gels from *D. pteronyssinus* (G) purified feces (red), (H) purified bodies (green) and (I) the overlay of

both. Yellow spots correspond to proteins present in both compared samples. White and blue arrows indicate main spots corresponding to group 1 and group 2 major allergens, respectively.

FIGURE 1

A



B

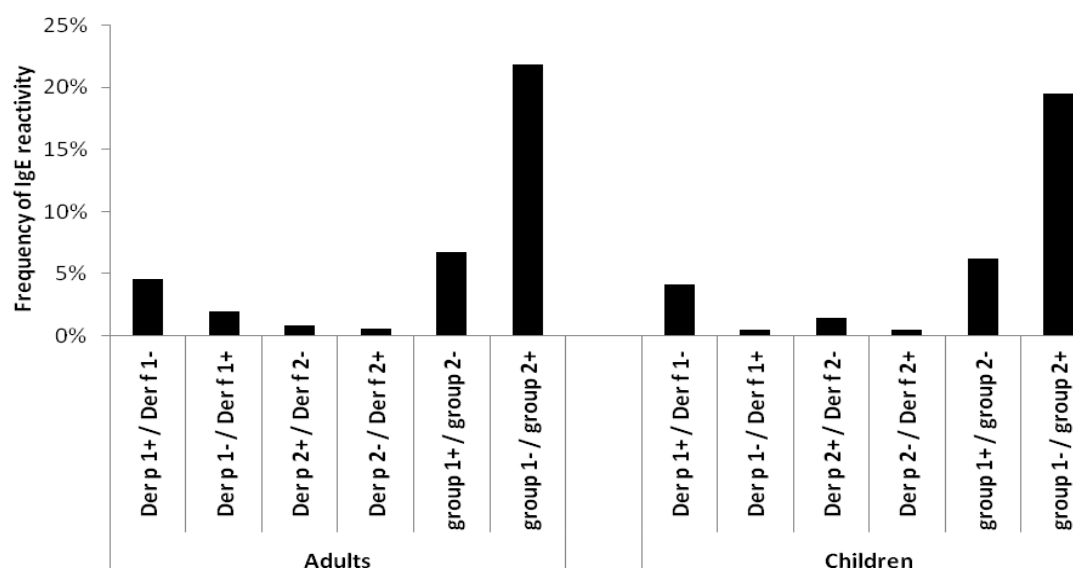
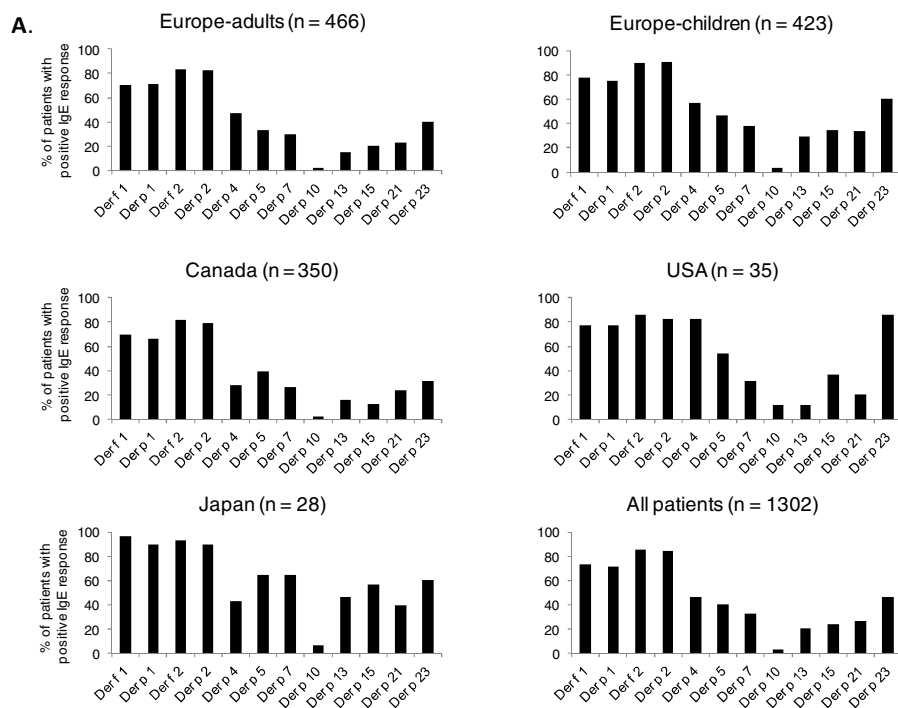


FIGURE 2



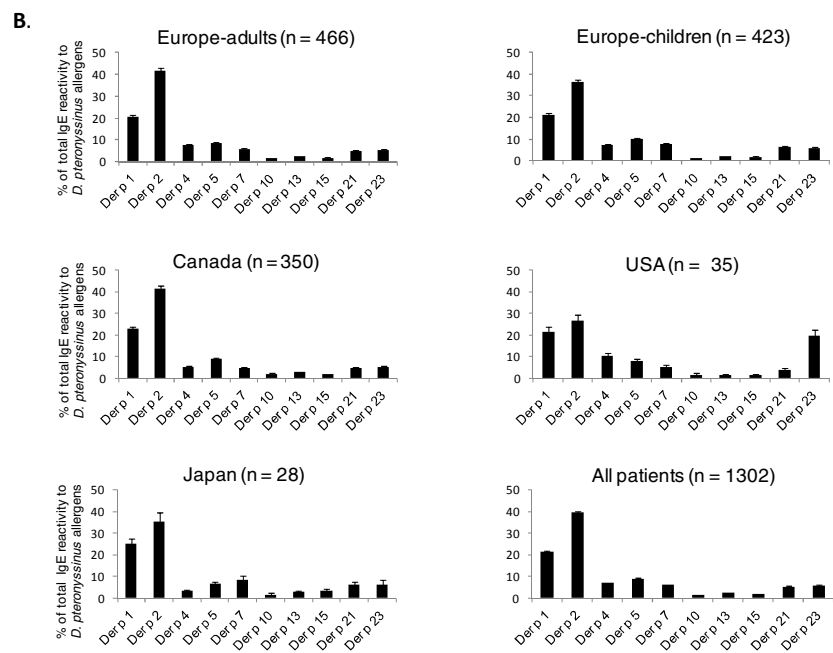


FIGURE 3

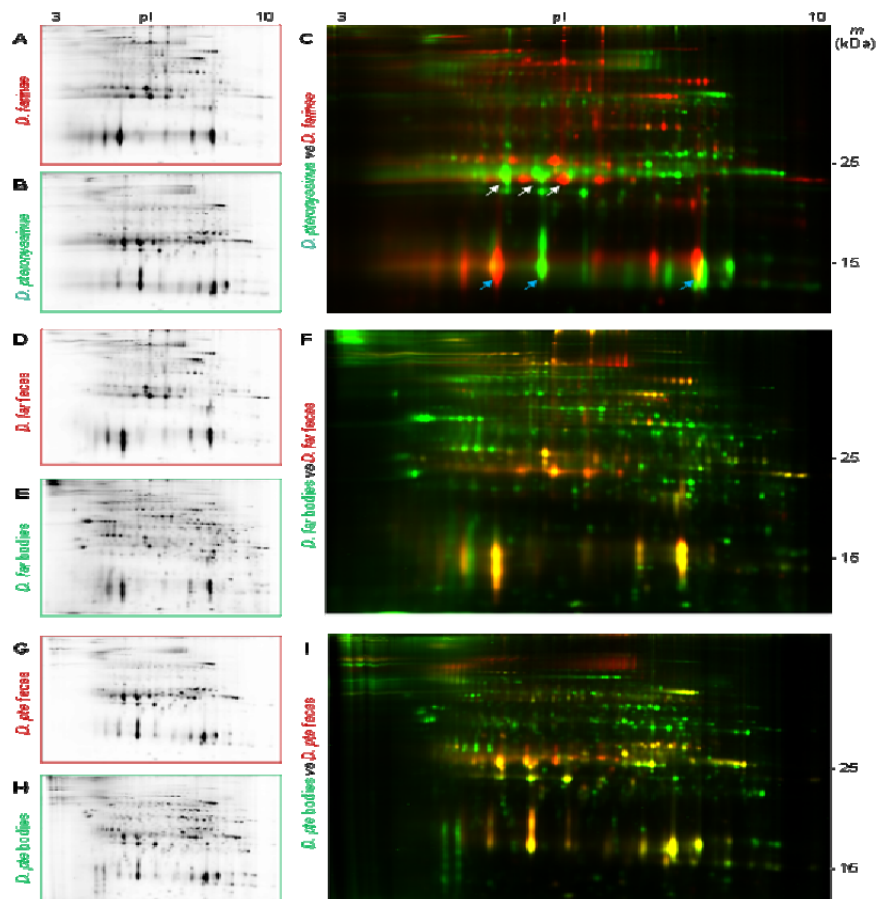


Table 1 Summary of MS/MS identification of allergens recorded by IUIS within whole extracts, purified bodies or feces from *D. pteronyssinus*

Allergen group	Isoallergen	Avg. mass (kDa)	Whole extracts		Bodies		Feces	
			Coverage (%)	#Peptides	Coverage (%)	#Peptides	Coverage (%)	#Peptides
1	Der p 1.0101	36	48	53	42	23	50	38
2	Der p 2.0101	16	64	23	61	13	71	17
3	Der p 3.0101	28	62	24	53	12	62	15
4	Der p 4.0101	57	77	29	43	14	36	13
5	Der p 5.0101	17	12	1	n.a.	n.d.	n.a.	n.d.
6	Der p 6.0101	30	72	39	54	14	71	21
7	Der p 7.0101	24	40	25	5	1	5	1
8	Der p 8.0101	26	18	4	13	3	n.a.	n.d.
9	Der p 9.0101	29	34	14	30	7	31	9
10	Der p 10.0101	33	18	5	28	11	n.a.	n.d.
11	Der p 11.0101	102	n.a.	n.d.	23	18	n.a.	n.d.
14	Der p 14.0101	191	7	9	23	36	n.a.	n.d.
15	Der p 15.0101	61	47	21	n.a.	n.d.	n.a.	n.d.
18	Der p 18.0101	52	42	16	11	3	26	6
20	Der p 20.0101	40	25	7	39	13	n.a.	n.d.
21	Der p 21.0101	17	18	2	n.a.	n.d.	n.a.	n.d.
23	Der p 23.0101	10	44	8	21	2	57	6

Avg. mass, theoretical average molecular mass of the allergen; Coverage, allergen amino acid sequence coverage; n.a., not applicable; n.d., not detected; #Peptides, number of peptides identified. Data shown are compiled from the analysis of 3 batches of whole extracts, and 1 batch each of purified bodies or feces. Der p x.0101 isoallergens were selected as comparators in order to determine sequence coverage, even if the presence of multiple isoallergens was detected for most allergens. Numbers of peptides identified by MS are only partially indicative of the abundance of corresponding allergens in the extracts. IgE reactivity of 2D blots of *D. pteronyssinus* extracts using a pool of sera from mite-allergic patients confirmed allergen integrity (data not shown).

Table 2 Summary of MS/MS identification of allergens recorded by IUIS within whole extracts, purified bodies or feces from *D. farinae*

Allergen group	Isoallergen	Avg. mass (kDa)	Whole extracts		Bodies		Feces	
			Coverage (%)	#Peptides	Coverage (%)	#Peptides	Coverage (%)	#Peptides
1	Der f 1.0101	36	61	50	46	20	63	69
2	Der f 2.0101	14	88	26	71	15	78	16
3	Der f 3.0101	28	64	25	45	9	68	17
6	Der f 6.0101	31	34	14	22	6	31	10
7	Der f 7.0101	24	42	26	53	14	n.a.	n.d.
8	Der f 8.0101	23	26	5	39	6	n.a.	n.d.
10	Der f 10.0101	35	33	9	48	27	n.a.	n.d.
11	Der f 11.0101	81	n.a.	n.d.	36	28	n.a.	n.d.
13	Der f 13.0101	15	n.a.	n.d.	31	5	n.a.	n.d.
14	Der f 14.0101	41	21	6	53	18	n.a.	n.d.
15	Der f 15.0101	63	46	27	37	15	46	22
16	Der f 16.0101	55	n.a.	n.d.	20	10	n.a.	n.d.
18	Der f 18.0101	52	48	18	16	6	8	5
20	Der f 20.0101	40	12	6	48	21	n.a.	n.d.
21	Der f 21.0101	16	24	6	32	3	n.a.	n.d.
22	Der f 22.0101	17	49	23	37	7	30	8
25	Der f 25.0101	27	34	5	29	4	6	1
26	Der f 26.0101	18	7	1	69	17	n.a.	n.d.
27	Der f 27.0101	48	21	6	3	1	3	1
28	Der f 28.0101	72	12	7	12	6	5	3
29	Der f 29.0101	18	40	4	52	8	9	1
30	Der f 30.0101	20	49	5	57	9	n.a.	n.d.
31	Der f 31.0101	17	21	2	50	8	n.a.	n.d.
32	Der f 32.0101	34	10	2	51	9	n.a.	n.d.
33	Der f 33.0101	52	n.a.	n.d.	3	1	n.a.	n.d.

Avg. mass, theoretical average molecular mass of the allergen; Coverage, allergen amino acid sequence coverage; n.a., not applicable; n.d., not detected; #Peptides, number of peptides identified. Data shown are compiled from the analysis of 3 batches of whole extracts, and 1 batch each of purified bodies or feces. Der f x.0101 isoallergens were selected as comparators in order to determine sequence coverage, even if the presence of multiple isoallergens was detected for most allergens. Numbers of peptides identified by MS are only partially indicative of the abundance of corresponding allergens in the extracts. IgE reactivity of 2D blots of *D. farinae* extracts using a pool of sera from mite-allergic patients confirmed allergen integrity (data not shown).

Patterns of IgE sensitization in house dust mite-allergic patients: implications for allergen immunotherapy

T. Batard¹, V. Baron-Bodo¹, A. Martelet¹, M. Le Mignon¹, P. Lemoine¹, K. Jain¹, S. Mariano¹, S. Horiot¹, H. Chabre¹, C. Harwanegg², C.A. Marquette³, B.P. Corgier³, W. T. Soh⁴, P. Satitsuksanoa⁴, A. Jacquet⁴, F-T Chew⁵, E. Nony¹, P. Moingeon¹

¹ Research and Development, Stallergenes, Antony, France

² Thermo Fisher Scientific ImmunoDiagnostics / Phadia Austria GmbH, Vienna, Austria

³ AXO Science, Villeurbanne, France

⁴ Chulalongkorn University, Bangkok, Thailand

⁵ National University of Singapore, Singapore

Correspondence

Philippe Moingeon, Research and Development, Stallergenes, 6 rue Alexis de Tocqueville, 92183 Antony Cedex, France. Tel.: 33.(0)1.55.59.49.57 - Fax: 33.(0)1.55.59.21.02
Email: pmoingeon@stallergenes.com

Supplementary Methods

Purification and characterization of mite allergens

Natural (n) Der p 1, Der f 1 and recombinant (r) Der p 7 used for coating on the multiplexed *HIFI Allergy* mite microarray were purchased from Indoor Biotechnologies. nDer p 2 and nDer f 2 were purified from *D. pteronyssinus* and *D. farinae* extracts, respectively, as previously described [6]. nDer p 4 was purified by affinity from a *D. pteronyssinus* extract using a column packed with cyclodextrin (Sigma, St Louis, Missouri) covalently bound to epoxy-activated sepharose 6B (GE Healthcare, Velizy, France) [1]. rDer p 5, rDer p 13, rDer p 15, rDer p 21 and rDer p 23 were produced in *Pichia pastoris* as described elsewhere [2]. rDer p 10 was expressed as a (His)₆-tagged molecule in the BL21(DE3) pLysS *E. coli* strain using the prokaryotic pET-Champion expression vector (Merck-Novagen, Fontenay-Sous-Bois, France), then purified following bacteria sonication by using a Ni²⁺ sepharose high performance column (GE Healthcare).

Concentrations of purified allergens were determined using either the Bradford assay (for natural proteins) or the micro bicinchoninic acid protein assay kit (Thermo Scientific, Illkirch, France) for recombinant molecules. SDS-PAGE analyses confirmed that all purified natural or recombinant HDM allergens exhibited the expected size, with a purity >80%, as measured with the Image Quant TL software (GE Healthcare), (Fig. S1). The identity of each allergen was confirmed by liquid chromatography combined with tandem mass spectrometry analyses (LC-MS/MS).

Purification of mite bodies and feces

Freshly harvested mite cultures were left to sediment in water in a separating funnel, in order to eliminate components from the spent culture medium as well as other particles (feces, mite debris). Mite bodies were recovered and allowed to dry on absorbent paper. Since mite feces are particles with a 10 to 50-μm diameter, whereas mite bodies are 165 to 350-μm long, feces were separated by sequential sieving of *D. pteronyssinus* and *D. farinae* cultures on 315-μm down to 100-μm stainless steel metal sieves. Feces were recovered as material passing through the last sieve. Body and feces purifications were confirmed by examination under an optical microscope.

Multiplexed mite microarray (*HIFI Allergy*)

Purified natural Der p 1, Der f 1, Der p 2, Der f 2, Der p 4 molecules and recombinant Der p 5, Der p 7, Der p 10, Der p 13, Der p 15, Der p 21, Der p 23 allergens were spotted on TKL 96-well plates (AXO Science) at a 300 µg/mL concentration as 650 pL drops using a piezoelectric spotter (sciFLEXARRAYER S3, Scienon, Germany). Human IgEs (AbCam, Paris, France) and human IgGs (Sigma-Aldrich, Saint Quentin Fallavier, France) were also spotted as positive and negative controls, respectively. Wells containing microarrays were saturated for 10 min at 37°C with 100 µL LowCross Buffer (Candor Bioscience, Wangen, Germany) prior to adding 25 µL of serum samples diluted 1:5 with LowCross Buffer for 120 min at 37°C. Wells were washed with PBS (AppliChem, Paris, France), and 100 µL of 2 µg/mL anti-human IgE labelled with alkaline phosphatase (Sigma-Aldrich) were added for 60 min at 37°C. Wells were further washed with PBS and 100 µL of a 5-bromo, 4-chloro, 3-indolylphosphate/nitro-blue tetrazolium substrate solution were added for 20 min. Wells were then washed with MilliQ water, dried at 50°C for 5 min, and analysed using a Colorimetric Image Reader (CLAIR, Sensovation, Radolfzell, Germany) with the AXOware 1.3.7 automated software. Signal intensities for each spot were calculated as the median intensity of all pixels included in a circular feature defining the spot. Results were expressed in IAUs (Intensity Arbitrary Unit) determined from the intensity of each spot of the microarray. The cut points for positivity of the assays were defined for each allergen as the global mean of background signals obtained, with negative controls calculated for each of the 16 plates as means + 3 SD of signals obtained for 4 negative controls within each plate. A serum sample was considered positive for IgE sensitization to a given allergen when the corresponding signal was above the cut point.

2D-DiGE comparisons of proteomes from *D. pteronyssinus* and *D. farinae* extracts, bodies and feces

Proteins from either whole source materials, bodies or feces from *D. pteronyssinus* and *D. farinae* were extracted with 50 mM ammonium bicarbonate for 24 h at +4 °C using a 1:10 (w:v) ratio for source materials and bodies, and a 1:6 (w:v) ratio for feces. Proteins were precipitated (using TCA, acetone, 10 mM dithiothreitol), solubilized (with 7 M urea, 2 M thiourea, 4% Chaps and 30 mM Tris, pH 8.8) and quantified with a Bradford assay. Samples were labeled with fluorescent CyDye (FluoProbes, Interchim, Montluçon, France) using a ratio of 8 pmoles of

dye/μg protein. Isoelectrofocusing was performed using an IPGphor 3 apparatus (GE Healthcare) with a voltage up to 10 000 V for a total of 56094 V.h. Focused proteins were reduced with dithiothreitol and alkylated with iodoacetamide. SDS-PAGE were subsequently performed on 12.5% polyacrylamide gels overnight at 10 mA/gel (Ettan Twelve, GE Healthcare) and gels were scanned using an Ettan DiGE Imager (GE Healthcare).

MS identification of *D. pteronyssinus* and *D. farinae* allergens and culture medium-derived proteins

Allergens from either whole source materials (3 batches), bodies or feces (1 batch each) from *D. pteronyssinus* and *D. farinae* were extracted as mentioned above. Additional extractions were performed for source materials using ethanol-formic acid according to Freiwald and Sauer's protocol (ref: Freiwald, A.; Sauer, S., Phylogenetic classification and identification of bacteria by mass spectrometry. Nat Protoc 2009;4(5):732-42). Protein content was measured in extracts with the Bradford assay, using bovine serum albumin (Thermo Scientific) as a standard. For enzymatic digestion, 5 μg proteins were denatured with 0.5% (w/v) RapiGest SF (Waters, Guyancourt, France) and disulfide bonds were chemically reduced using dithiothreitol and alkylated by iodoacetamide. Trypsin digestion was performed at 37°C for 3 h using a final protein: trypsin ratio of 25:1, then reactions were quenched by adding 1 M HCl. Digested samples were submitted to nanoLC-MS/MS analysis as follows. Samples (0.5-1 μg proteins) were loaded onto an Acclaim PepMap 100 C₁₈ Trap column (Thermo Scientific) and separated at 50°C using an Acclaim PepMap RSLC C₁₈ column (2 μm, 100 Å, 75 μm x 50 cm, Thermo) using an Ultimate 3000 RSLCnano liquid chromatography system (Thermo). The aqueous mobile phase (A) consisted of HPLC-grade water with 0.1% formic acid, while the organic phase (B) comprised 80% acetonitrile with 0.1% formic acid. A 200-min gradient profile was used at a flow rate of 300 nL/ and the column eluent was introduced into a QqToF mass spectrometer (Impact HD, Bruker Daltonics, Wissembourg, France) equipped with a CaptiveSpray source. Analyses were performed in the positive ion mode using capillary and end plate offset voltages of 1300 V and 500 V, respectively. The dry temperature was set at 180 °C. Data were acquired by the ToF analyzer from m/z 50 to 2000. MS/MS spectra were acquired in an intensity dependent mode at a rate of 2-16 Hz with a maximum of 20 precursor ions in the range of m/z 300-1800 (charge state >2) and a MS spectra rate of 2 Hz. Mass calibration was performed both externally using the ESI-

L Low Concentration Tuning Mix (Agilent Technologies, Les Ulis, France) as well as internally using the lock mass option in MS mode: m/z 1221.9906 (Chip cube high mass reference, Agilent Technologies).

HDM allergen identifications within whole extracts, purified bodies or feces were performed by matching acquired peptide tandem mass spectra with theoretical digests gathered in a mite allergen database (including 281 entries downloaded on 2015-03-30 from IUIS, UniProt and Allergome). Moreover, in-house transcriptome derived proteome databases of *D. pteronyssinus* (143474 entries) and *D. farinae* (20154 entries) supplemented with both *Triticum aestivum* and *Saccharomyces cerevisiae* databases from UniProt (195654 entries) were used in order to identify proteins from mites as well as yeast and wheat.

Database search was performed using the PEAKS software v7 (Bioinform, Waterloo, Canada) with the following parameters: peptide tolerance of 10 ppm (0.05 Da for fragment ions), trypsin enzyme, non-specific cleavage at both ends of the peptide allowed (only for allergen identifications), one missed cleavage permitted, carbamidomethylation of cysteine as a fixed modification, variable modifications (e.g. deamidation of asparagine and glutamine, oxidation of methionine), allowing a maximum of 1 or 5 variable post-translational modifications for allergen or mite, wheat and yeast protein identifications, respectively. Protein identifications were confirmed with the application of a 0.1% peptide-spectrum matches false discovery rate (estimated with the decoy fusion method) and visual inspection of peptide MS/MS data or a 1% peptide sequence matches false discovery rate and 1 unique peptide for *Triticum aestivum* and *Saccharomyces cerevisiae* proteomes.

MS quantification of group 2 allergens from *D. pteronyssinus* and *D. farinae*

A LC-MS/MS assay was developed in order to quantify group 2 allergens in a comprehensive manner. This assay relies on a standard addition method using a protein reference standard (i.e. purified recombinant Der p 2) representative of the group 2 allergens to be quantified. Briefly, samples were spiked with various amounts of the purified rDer p 2 standard protein, reduced, alkylated and digested with trypsin. Two house dust mite group 2 allergen representative peptides were quantified by LC-MS/MS using 3 transitions per peptide. Group 2 allergen (i.e. Der f 2 and Der p 2) contents were determined based on the average value of those 2 representative peptides.

Dot blot analyses of mite allergens in *D. farinae* extracts

Purified natural Der f 3, Der f 6, Der f 9, as well as recombinant Der f 4, Der f 5, Der f 7, Der f 8 and Der f 13 (all from National University of Singapore, Singapore) were loaded at 1 µg/mL on nitrocellulose membranes as positive controls, and 1 µg of bovine serum albumin as a negative control. Membranes were air-dried and blocked with phosphate buffered saline (PBS)-0.1% Tween-20 and incubated with allergen-specific rabbit polyclonal antibodies (1/2 v/v in PBS) at +4°C overnight, followed by 2 hours with anti-rabbit antibodies conjugated with alkaline phosphatase (1/1000 v/v in PBS; Sigma-Aldrich). Membranes were revealed with NBT/5-bromo-4-chloro-3-indolyl-phosphate (Promega) for 30 minutes and spot intensities measured with an imaging software (Microimage v.3.01).

Supplementary References

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2. Pulsawat P, Theeraapisakkun M, Nony E, Le Mignon M, Jain K, Buaklin A, *et al.* Characterization of the house dust mite allergen Der p 21 produced in *Pichia pastoris*. *Prot Expr Purif* 2014;**101**:8-13.

SUPPLEMENTARY FIGURE LEGENDS

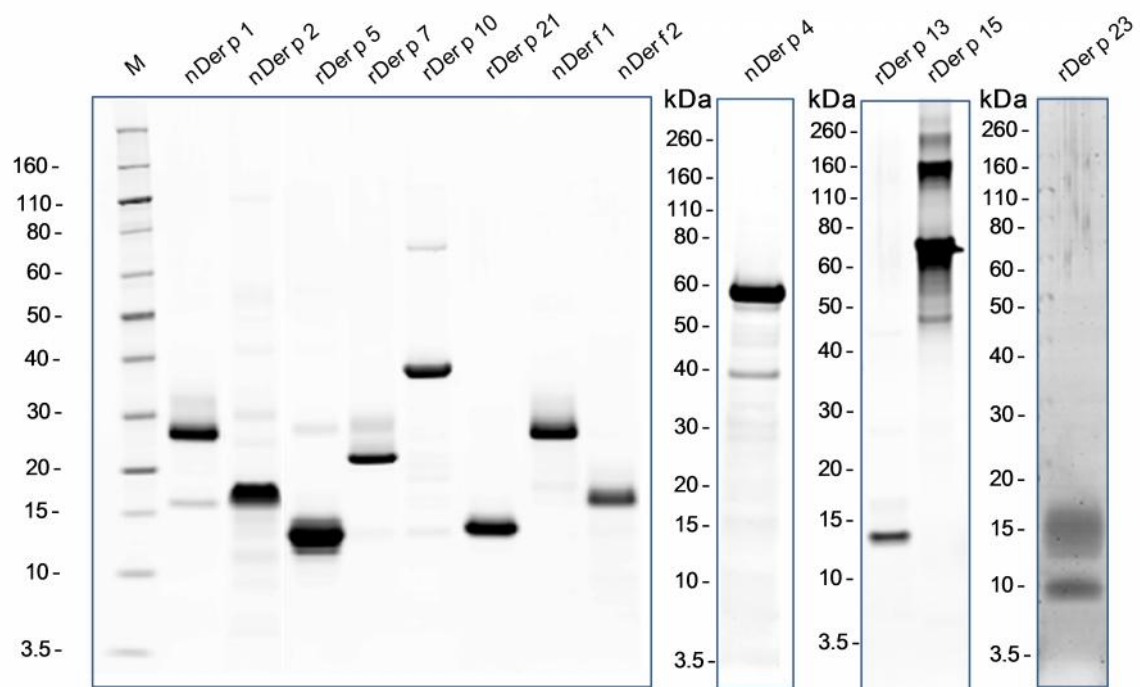
SUPPL FIGURE 1: SDS PAGE analysis of purified allergens used in the *HIFI Allergy* customized multiplexed mite microarray

Natural or recombinant purified allergens were obtained as described in methods. Following SDS-PAGE on 4–12% Bis-Tris acrylamide gels under reducing conditions, proteins were stained with Sypro Ruby. The purity of the allergens measured as described in methods, ranges from 80 to 100%. Multimers are detected for Der p 15. MS analysis of Der p 23 confirmed that the 2 bands are related to the allergen. M : molecular weight markers.

SUPPL FIGURE 2: Immunodot blot analysis of representative *D. farinae* extracts

Batches V2390 and V2527 of *D. farinae* extracts (lanes 1 and 2, respectively) were assayed for the presence of *Dermatophagoides*-specific group 3–10 and 13 allergens using specific rabbit polyclonal antibodies raised against each individual recombinant proteins. Purified natural or recombinant mite allergens and bovine serum albumin (both used at 0.1 µg/dot) were included as positive and negative controls (lanes 3 and 4, respectively). Each allergen was adjusted to a 250-µg/ml protein concentration, with 1 µl spotted on each dot. Using allergen concentrations curves, we estimate that for a 5% (m/v) allergen extract, “-” corresponds to allergen levels below the detection limit of ~0.60 µg/mL, “+” to levels between ~0.6 and ~5 µg/mL, “++” to levels between ~5 and ~20 µg/mL, and “+++” to levels above ~20 µg/mL.

SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2

				Allergen	Der f (batch V2390)	Der f (batch V2527)
	1	2	3	4		
Der f 13					Der f 13	++
Der f 10					Der f 10	++
Der f 9					Der f 9	++
Der f 8					Der f 8	+++
Der f 7					Der f 7	++
Der f 6					Der f 6	+
Der f 5					Der f 5	++
Der f 4					Der f 4	+++
Der f 3					Der f 3	++

198 **Table S1.** Correlations between allergen-specific IgE levels measured by using either ImmunoCAP, ISAC assay, or *HIFI Allergy*
 199 customized multiplexed microarray

Allergen	Der f 1	Der f 2	Der p 1	Der p 2	Der p 5	Der p 7	Der p 10
	n = 786	n = 786	n = 815	n = 815	n = 368	n = 368	n = 815
Correlations between IgE levels measured by <i>HIFI Allergy</i> and ISAC	r = 0.86	r = 0.71	r = 0.85	r = 0.72	r = 0.84	r = 0.81	r = 0.26
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
			n = 374	n = 374			
Correlations between IgE levels measured by <i>HIFI Allergy</i> and ImmunoCAP			r = 0.89	r = 0.79			
			$p < 0.0001$	$p < 0.0001$			

200
 201 Spearman correlation analyses between allergen-specific IgE titers measured using either the mite microarray or commercial assays
 202 such as ISAC or ImmunoCAP were established by testing randomly selected serum samples from 815to 374 HDM-allergic patients,
 203 respectively.